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Identification of protein components reactive with anti-PM/Scl autoantibodies

C. GELPI, A. ALGUERÓ, M. ANGELES MARTINEZ, S. VIDAL, C. JUAREZ &
J. L. RODRIGUEZ-SANCHEZ *Department of Immunology, Santa Cruz y San Pablo Hospital, Barcelona, Spain*

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SUMMARY

The PM/Scl antigen from mammalian cells has been characterized as a nucleolar and nucleoplasmic molecular complex containing at least 16 polypeptides ranging in molecular weight from 110 to 20 kD. Of these polypeptides, we have found those of 68, 39 and 20 kD to be in a phosphorilated form. Whereas the entire complex was precipitated by all the anti-PM/Scl sera tested, in immunoblots the antibodies specifically recognized determinants on the 110-kD protein. This protein was immunoprecipitated more preferentially from nucleoli extracts than from total cell extracts. Moreover, this protein disappeared from the immunoprecipitates when treated with DNAse. Likewise, the immunoblot reaction of the specific antibodies with the 110-kD protein was abolished by treatment of the extracts with DNAse and trypsin, and was resistant when extracts were treated with RNAse. Affinity-purified antibodies from this protein selectively stained the nucleoli and the nucleoplasm of the mammalian cells. Moreover, when the cultured cells used in immunofluorescence were treated with DNAse, the affinity purified antibodies from the 110-kD protein gave negative fluorescence. However, when whole anti-PM/Scl sera were used, a nucleolar and nucleoplasmic staining was found. We conclude that the 110-kD protein has at least one of the autoimmunogenic epitopes of the PM/Scl antigen, recognized by all anti-PM/Scl sera tested. Other epitopes differing in their DNAse sensitivity may also be present in the PM/Scl antigen.

Keywords PM/Scl polymyositis/scleroderma anti-nuclear anti-nucleolar autoantibodies

INTRODUCTION

Autoantibodies reactive with the nucleolus are found in a variety of rheumatic diseases (Tan, 1989) and are specifically common among patients with scleroderma (Reimer *et al.*, 1988), polymyositis and scleroderma–polymyositis overlap syndrome.

Nucleoli are complex structures engaged in the assembly of pre-ribosomal particles. They contain DNA segments which specify ribosomal RNA, several types of small nuclear ribonucleoprotein particles (snRNP) such as the U3 and Th particles (Hashimoto & Steitz, 1983; Reddy *et al.*, 1983; Lischwe *et al.*, 1985), and a very large array of proteins (Douvas, Achten & Tan, 1979; Stetler *et al.*, 1982; Shero *et al.*, 1986; Rodriguez-Sanchez *et al.*, 1987). Among these, the PM/Scl antigen has been described as a nucleolar antigen recognized by 8% of patients with polymyositis and dermatomyositis (Reichlin *et al.*, 1984; Targoff & Reichlin, 1985). There has been a report on the presence of anti-PM/Scl antibodies in serum from five of 161 (3%) patients with systemic sclerosis and four of 36 (11%) patients with myositis; the four patients with myositis had also signs of scleroderma (Bernstein, Bunn & Hughes, 1984). Reimer *et al.* (1986) reported that the antigen is a complex structure of at least 11 polypeptides, but its reactive epitopes have not yet been recognized.

Here we describe the protein component reactive with the anti-PM/Scl antibodies and give more information about the macromolecular characterization of the antigen and its cellular implications probably with pre-ribosomal complex components.

Correspondence: C. Gelpi, Department of Immunology, Santa Cruz y San Pablo Hospital, 08025 Barcelona, Spain.

MATERIALS AND METHODS

Sera

The sera studied were obtained from 127 patients with various rheumatic diseases: 72 corresponded to progressive systemic scleroderma, 12 polymyositis, 23 sclero-polymyositis, 10 rheumatoid arthritis and 10 systemic lupus erythematosus. Ten normal sera from donor blood volunteers were used as control. Anti-PM/Scl reference sera were kindly provided by Dr E. Tan.

Preparation of cell extracts

HeLa and FLC (Friend erythroleukaemia) cells were maintained at 37°C, 5% CO_2 growing in log phase at 2×10^5 and 4×10^8 cells/ml, respectively, in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% glutamine, 100 U/ml penicillin and 60 μg/ml streptomycin (GIBCO, Chagrin Falls, OH).

Whole-cell extracts were prepared as described previously (Forman *et al.*, 1985).

Purified nucleoli were prepared as described by Olson *et al.* (1974) and nuclear chromatin isolated as described by Matsui, Fuke & Busch (1977).

Soluble fractions of nucleolar chromatin were prepared by overnight extraction, stirring the pellet of nucleolar chromatin in 10 mM Tris-HCl, pH 8, and 2 mM PMSF. The clarified cell supernatant was prepared by centrifugation at 14000 *g* for 20 min at 4°C and used as a source of antigen.

Analysis of immunoprecipitated proteins

A procedure based on that of Matter *et al.* (1962) was used with some minor modifications (Mimori, Hardin & Steitz, 1986). HeLa and FLC (Friend erythroleukemia) cells growing in log phase at 2×10^5 and 4×10^5 cell/ml, respectively, were allowed to incorporate ^{35}S-methionine (5 μCi/ml; Amersham, Arlington Heights, IL) in methionine-free minimal essential medium supplemented with 1% glutamine and antibiotics for 16–18 h. For phosphorilated protein analysis, cells were labelled with ^{32}P-orthophosphate (10 μCi/ml; New England Nuclear, Boston, MA) in 100 ml of phosphate-free minimal essential medium for 14–16 h.

For DNAse digestion experiments, immunoprecipitates were resuspended in Tris-buffered saline (TBS; 130 mM NaCl, 40 mM Tris-HCl, pH 7·4) containing 5 mM $MgCl_2$, 0·2 mM PMSF and treated with DNAse I (Miles Laboratories, Slough, UK) (0·1 mg/ml) for 30 min at 37°C. The digestion was stopped with 2 mM EDTA. After three washes with immunoprecipitation buffer (IPP; 10 mM Tris-HCl, pH 8, 0·5 M NaCl, 0·1% Nonidet P-40), the bound proteins were extracted and analysed electrophoretically.

Immunoblots

Immunoblots were performed as described previously (Towbin, Staehelin & Gordon, 1979) using 1% haemoglobin, 1% gelatin (Ria Grade, Sigma Chemicals Co., St Louis, MO) as blocking solution and ^{125}I-protein A (Amersham) for detection of bound immunoglobulins.

The substrates used for protein blots were total HeLa and FLC cell extracts, isolated nucleoli, and nucleolar chromatin fraction prepared as described previously.

For enzyme digestion experiments, aliquots of cell extracts were digested with DNAse I (Miles Laboratories) (0·1 mg/ml) containing 5 mM $MgCl_2$, 0·2 mM PMSF, or RNAse (Sigma) (0·1 mg/ml), or trypsin (Sigma) (0·2 mg/ml) for 1 h at 37°C.

Immunoaffinity purification of antibodies

Antibodies were affinity-purified as described by Olmsted (1981) and Krohne *et al.* (1982), with minor modifications: nitrocellulose paper-bound antibodies were eluted with 0·2 M glycine-HCl at pH 2·5. Eluates were neutralized immediately and dialysed against TBS containing 0·2 mM PMSF. Affinity-purified antibodies were used without dilution in indirect immunofluorescence studies, immunoblot assays and immunoprecipitation experiments.

Preparation of cells for indirect immunofluorescence

Hep-2, HeLa and Vero cells grown on glass slides were fixed as described (Weber, Bibring & Osborn, 1975), washed for 5 min in 0·5% Triton X-100 in PBS and 5 min in PBS and used as substrate. The fluorescein-labelled anti-human immunoglobulin conjugate was purchased from Dako Laboratories (Santa Barbara, CA), and used at a dilution of 1:50.

Information concerning the nature of the antigen in the cells was obtained by pretreatment with enzymes or chemical reagents to determine whether or not such treatment destroyed reactivity with antisera. Cells grown on glass slides were fixed with acetone at −20°C for 20 min and washed as above. The slides were then treated with DNAse I (Miles Laboratories) (40 U/ml in PBS), RNAse (Sigma) (0·1 mg/ml) for 60 min at 37°C or trypsin (Sigma) (100 U/ml in PBS), for 10 min at 37°C. Other slides were extracted with 0·1 M HCl, 0·1 M Tris-HCl pH 8, 0·1 M Tris-HCl pH 9, 1 M NaCl, or 2 M NaCl, for 30 min at room temperature and used immediately for indirect immunofluorescence.

Immunofluorescence studies were also performed on HeLa, Hep-2, and Vero cells treated with actinomycin D. In brief, tissue culture cells were incubated with RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) containing 10% FCS, 1% glutamine and 0·1 μg/ml actinomycin D for 4 h at 37°C. The cells were then washed with fresh medium, fixed and processed for indirect immunofluorescence staining as described above.

Preparation of chromosomes

Colcemid (GIBCO) was added to the culture medium of HMcB melanoma cells in a final concentration of 0·075 μg/ml. After 4 h, the cells were collected and chromosomes were prepared and used as substrate for indirect immunofluorescence as described in a previous paper (Rodriguez-Sanchez *et al.*, 1987).

RESULTS

Sera from nine patients with scleroderma/polymyositis (five) or progressive systemic scleroderma (four), were found to contain antibodies that uniformly immunoprecipitated 16 polypeptides from ^{35}S-methionine-labelled HeLa cell extract, also recognized by the reference sera (Table 1). The approximate molecular weight estimates were M_r 110, 100, 90, 80, 77, 73, 70, 68, 39, 37, 33, 30, 27, 26, 22, and 20 kD. An additional 18-kD polypeptide was also present when FLC extracts were used (Fig. 1). The anti-PM/Scl reference serum (lane 5) precipitated two extra proteins of M_r 41, and 52 kD not present in the immunoprecipitates of the other anti-PM/Scl sera, probably unrelated to the PM/Scl

Table 1.

Patient sera	ANAs	Diagnosis
1	1:320 N, S	Scleroderma
2	1:20480 N, S	Scleropolymyositis
3	1:5120 N, S	Scleroderma
4	1:20480 N, S	Scleroderma
5*	1:20480 N, S	
6	1:10240 N, S	Scleropolymyositis
7	1:5120 N, S	Scleropolymyositis
8	1:5120	Scleropolymyositis
9	1:10240 N, S	Scleropolymyositis
10	1:5120 N, S, H	Scleroderma

* Reference sera kindly provided by Dr E. Tan.

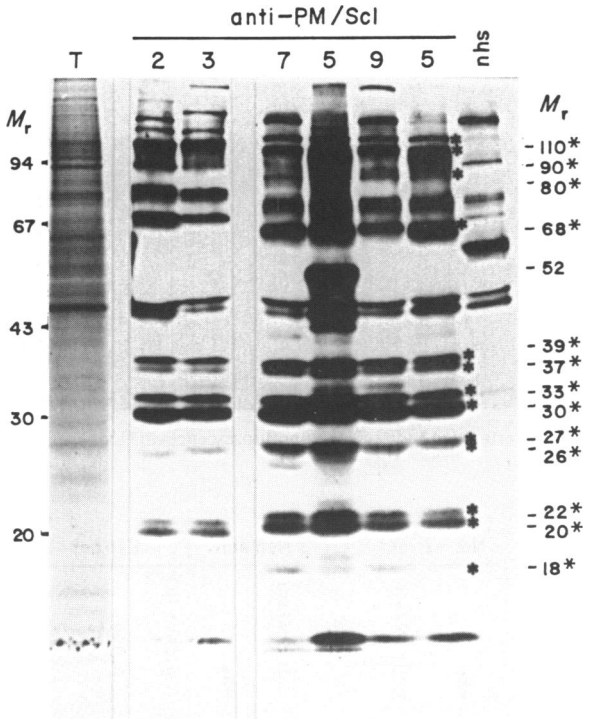

Fig. 1. Gel fractionation from ^{35}S-labelled proteins in immune precipitates. Nuclear sonicates were prepared from ^{35}S-labelled FLC cells. Immune complexes were recovered from a protein A-Sepharose, and the proteins were resolved in 10% SDS–PAGE. Control sera used were anti-PM/Scl reference serum, kindly provided by Dr E. Tan (lane 5) and a normal human serum (NHS). The numbers at the top of the Figs corresponded to the anti-PM/Scl sera from Table 1.

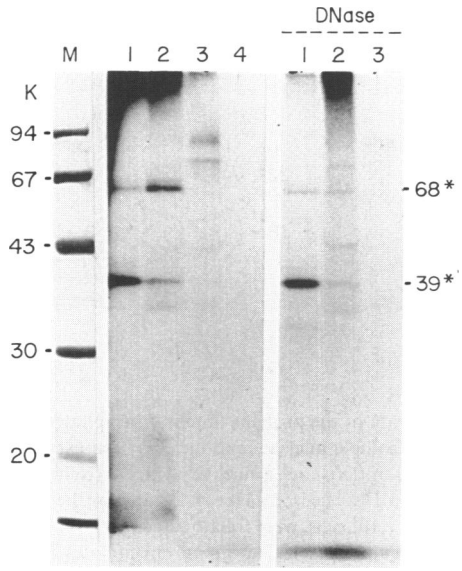

Fig. 2. Fractionation of proteins from a ^{32}P orthophosphate-labelled proteins in immune precipitates of FLC cell extracts. Lanes 1 and 2 show the immunoprecipitates of anti-PM/Scl sera no. 7 and 9 from Table 1; lanes 3 and 4 show the immunoprecipitates of control sera anti-NOR:90 and a normal human serum, respectively. On the right, the DNAse treated immunoprecipitates are shown.

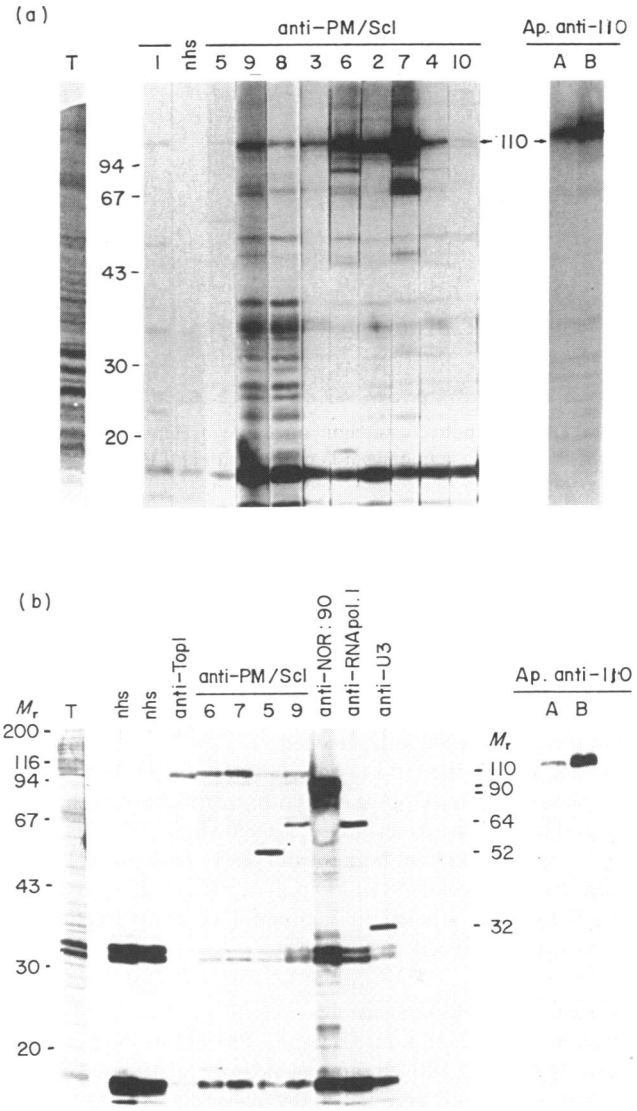

Fig. 3. Identification of PM/Scl polypeptides recognized by sera with anti-PM/Scl antibodies. The equivalent to 10^7 FLC cells were fractionated in each lane of 10% polyacrylamide-SDS gels and electrophoretically transferred to nitrocellulose paper. Panel (a) contains whole cell extract; and panel (b) contains nucleolar chromatin. The amido black-stained substrates are shown on the left of each panel. Sera tested were the patient sera (from Table 1) with anti-PM/Scl antibodies, additional scleroderma patient sera containing antibodies against known nucleolar antigens, and normal human sera. Lanes 'a.p. anti-110') show the immunoblot analysis of the affinity-purified antibodies anti-110 kD protein. Lanes a were probed with affinity-purified antibodies from nuclei extracts, and lanes b, from nucleoli. Bound antibodies were detected with ^{125}I-protein A.

antigen. The 110-kD protein was more evident in the FLC cell extracts than in the HeLa cell extracts. Those proteins of 68 and 39 kD were in a phosphorilated form (Fig. 2). An additional 20-kD protein was found in a phosphorilated form, using FLC extracts.

After DNAse I digestion of the immunoprecipitates performed with anti-PM/Scl sera and ^{35}S-methionine-labelled cell extracts, the 110-, 100-, 90-, 80- and 20-kD proteins disappeared from the immunoprecipitates.

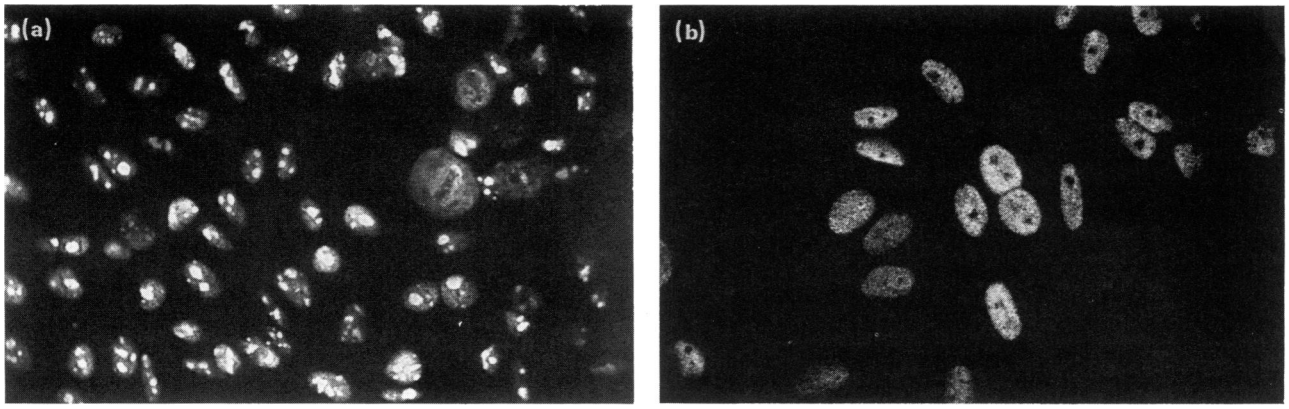

Fig. 4. (a) Indirect immunofluorescence on Hep-2 cells stained with anti-PM/Scl serum. (b) Indirect immunofluorescence on Hep-2 cells treated with actinomycin D probed with anti-PM/Scl serum (no. 3 from Table 1).

Characterization of the reactive polypeptide

All the anti-PM/Scl sera were studied by immunoblots using HeLa and FLC extracts. Each of these sera recognized a single polypeptide of approximately 110 kD in total cell extracts, isolated nucleoli and nucleolar chromatin extract (Fig. 3a, b). Other polypeptides reactive with anti-PM/Scl sera were seen, but only the 110 kD was present in all the immunoblot reactions with the anti-PM/Scl sera screened.

Reactivity of the 110-kD polypeptide with anti-PM/Scl sera was abolished after DNAse or trypsin treatment of both whole cell and nucleolar extracts (data not shown).

Affinity-purified antibodies from the 110-kD protein recognized the same reactive 110-kD polypeptide (Fig. 3, lanes 'a.p. anti-110') when studied in immunoblots using fractionated nucleoli and total cell as substrates.

Indirect immunofluorescence

By indirect immunofluorescence, anti-PM/Scl antibodies recognized a cellular antigen homogeneously distributed in the nucleoli and weakly granular in the nucleoplasm (Fig. 4a). The cell cytoplasm also showed a weak fluorescence with a perinuclear localization. The staining was abolished when cultured cells were treated with 2 M NaCl, 0·1 M HCl, or Tris-HCl, pH 9. Moreover, this antigen was sensitive to trypsin, resistant to digestion with RNAse, and partially resistant to DNAse (the immunofluorescence was weaker than without the treatment). Negative fluorescence was seen by indirect immunofluorescence on isolated metaphase chromosomes.

To confirm that the 110-kD protein recognized in immunoblots by anti-PM/Scl sera is the antigen (or at least one of the antigens) responsible for the staining of the nucleoli and nucleoplasm, we employed the affinity-purified antibodies. Antibodies eluted from the 110-kD protein selectively stained the nucleoli in the typical homogeneous pattern as well as the nucleoplasm in a speckled pattern, similar to that provided by the whole sera. However, the staining was abolished when cells were treated with DNAse, but unaltered by treatment with RNAse (whole anti-PM/Scl sera, introduced in the experiment as a positive control, provided the weak positive nucleolar and nucleoplasmic staining, as stated above).

After treating the cultured cells with concentrations of actinomycin D capable of inhibiting the action of the RNA pol I, the antigen restructured itself and was observed in the

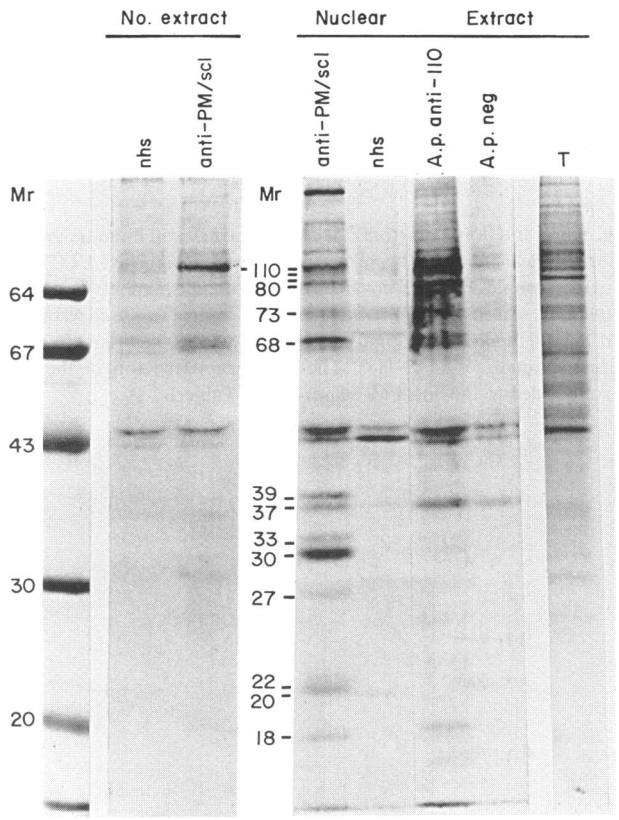

Fig. 5. Comparison of the proteins immunoprecipitated with anti-PM/Scl sera from a soluble nuclear and nucleolar cell extracts. A nucleoli chromatin fraction (left) and a nuclear sonicate (right) were prepared from ^{35}S-labelled FLC cells. In lane T, 3 μl from the nuclear extract, equivalent to 12×10^4 cells, were fractionated in a 10% polyacrylamide-sodium dodecyl sulphate gel. The immunoprecipitates with anti-PM/Scl serum, normal human serum and affinity-purified antibodies ('a.p.') were recovered from a protein A-Sepharose. Proteins were analysed on the fluorography of the gel. The molecular weights and position of polypeptide standards corresponding to the nucleolar extract immunoprecipitates are given on the left, and the molecular weight estimates of the PM/Scl polypeptides immunoprecipitated by the affinity-purified anti-110 antibodies in the middle.

nucleoplasm of the cells (Fig. 4b). The same results were found using whole serum or anti-110 kD affinity-purified antibodies.

Molecular characterization of the nucleolar component of the PM/Scl antigen

The observations that the PM/Scl antigen was predominantly localized in the nucleoli and somehow associated to DNA, prompted us to determine the proteins precipitated by the anti-PM/Scl sera from a nucleolar chromatin extract. We prepared ^{35}S-methionine-labelled immunoprecipitates both from whole FLC cell extracts and from nucleoli chromatin extracts. The anti-PM/Scl sera precipitated the characteristic array of polypeptides of the PM/Scl antigen from the FLC nuclear extract (Fig. 5, right). When nucleolar chromatin extract was used, a dramatic increase of the 110-kD protein relative to the other ^{35}S-methionine-labelled proteins was seen (Fig. 5, left).

A complementary approach for examining the molecular composition of the 110-kD antigen and its relation to the PM/Scl antigen was performed. If the 110-kD protein were associated to the PM/Scl antigen, all or at least some of the 17 polypeptides of PM/Scl would be expected to coprecipitate when anti-110 affinity-purified antibodies were used. Figure 5 (right) shows that the anti-110-kD antibodies affinity-purified from nucleolar extracts, precipitated the characteristic set of proteins with molecular weights ranging from 110 to 18 kD, with the exception of the 39, 22 and 20 kD components. Moreover, the 30-kD protein was very weakly precipitated. In addition, other proteins were precipitated, possibly unspecifically.

DISCUSSION

In the present study we identified nine patients whose sera contained antibodies which selectively recognized the PM/Scl antigen. All uniformly precipitated a complex of at least 16 proteins of approximate molecular weights ranging from 110 to 20 kD. An additional 18-kD protein was also present when FLC extracts were used. The experiments described in this paper clearly establish that all the anti-PM/Scl antibodies containing sera react specifically with epitopes localized in the 110-kD protein.

Studies performed with affinity-purified antibodies localized this antigen in the nucleoli and in the nucleoplasm of all the mammalian cells tested.

The 110-kD-reactive protein must interact with DNA, either directly or by association with another DNA-binding protein. This protein might be recognized by the antibody in the context of its interaction with DNA, as suggested by the negative results provided by the indirect immunofluorescence studies using DNAse-treated cells as substrates and the disappearance of the immunoblot reaction after DNAse treatment of the substrates used. However, when the immunofluorescence studies were performed with the whole serum anti-PM/Scl, a positive reaction was found in the nucleoli and nucleoplasm. Moreover, in immunoprecipitation experiments performed with anti-PM/Scl sera, the 110-kD protein disappeared from the immunoprecipitate after the DNAse digestion, but other proteins remained. These results suggest that different epitopes were recognized by the anti-PM/Scl antibodies.

When anti-110-kD antibodies, affinity-purified from either nuclear or nucleolar extracts, were used to perform the immunoprecipitation experiments, a relative enrichment of the high molecular weight components of the PM/Scl antigen relative to the other ^{35}S-methionine labelled proteins was found. Moreover, these antibodies failed to precipitate some of the low molecular weight components of the PM/Scl antigen. These results suggest that in its native state the PM/Scl antigen may exist as a complex or as associated particles.

From the complex set of proteins immunoprecipitated by the anti-PM/Scl antibodies, some were in a phosphorilated form. Reimer *et al.* (1986) reported the presence of 11 polypeptides, two of them of 80 and 20 kD in a phosphorilated form. We found two phosphorilated proteins of 68 and 39 kD in HeLa cell extracts, and an additional protein of 20 kD in FLC extracts. In addition, duplicated samples of the immunoprecipitates containing the phosphorilated proteins were treated with DNAse and RNAse respectively, and no changes in their molecular weight estimates were found. Bernstein, Bunn & Hughes, 1984, reported that the anti-PM/Scl antibodies immunoprecipitated three proteins of molecular weight 26, 31 and 36 kD. We believe that the discrepancy between previous data and this report may be due to differences in the procedure by which the extracts were prepared or due to differences in the HeLa cell strains used. In addition, Reimer *et al.*, 1986, reported that some of the anti-PM/Scl sera reacted with a protein of approximately 80 kD. We compared the blot assays using nuclear and nucleolar extracts separated in SDS–PAGE prepared as they reported. When 17·5 and 15% polyacrylamide gels were used, the 110-kD component of the PM/Scl antigen was not able to transfer to the nitrocellulose paper.

In a recent communication, Blutner, Gent & Baütz (personal communication) reported that sera from patients with scleroderma–polymyositis overlap syndrome (PM/Scl) recognized two major nucleolar proteins of 95 and 75 kD in Western blots. We believe that the differences in the molecular weight of the major protein carrying the epitopes recognized by these autoantibodies could be due to protease sensitivity of the 110-kD protein.

In this report, the immunoblot assay using whole nucleolar chromatin as substrate proves to be a simple method for easy identification of anti-PM/Scl antibodies in patients sera. Anti-PM/Scl antibodies may occur in patients with scleroderma or PM/Scl. Approximately 7·0% of sera from patients with rheumatic diseases screened in this study by our standard immunoblot assay recognized the PM/Scl 110-kD protein and immunoprecipitated the polypeptides of the antigen. Moreover, 5·5% of patients with scleroderma and 14% of patients with polymyositis had anti-PM/Scl antibodies in sera.

To date, there are few clues to the function of this antigen. The results of the distribution of the antigen after treatment with actinomycin D suggest it is associated with the synthesis or maturation machinery of ribosomes. However, its association to DNA, either directly or by union to a DNA-binding protein, points to the hypothesis that the autoimmune responses in patients with scleroderma select targets from a certain set of non-histone chromatin components that differ from the autoantigenic targets of other rheumatic diseases. Currently recognized autoantibodies associated with scleroderma and their variants include specificities for topoisomerase I (Shero *et al.*, 1986), several components of the chromosome centromere (McNeilage *et al.*, 1986), the Ku protein (Mimori, Hardin & Steitz, 1986) and the NOR:90 protein (Rodriguez-Sanchez *et*

al., 1987), all related to DNA. An understanding of why these autoimmune responses are so specifically directed to DNA-associated antigens could provide important insight into the basic pathogenetic mechanisms of scleroderma and related diseases.

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